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1. Your reference — **G021706PT/HGH**

2. Patent application number *(The Patent Office will fill in this part)* — 18 DEC 1998 — **9828000.1**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Chiron SpA
Via Fiorentina 1
53100 Siena
Italy

Patents ADP number *(if you know it)* 7157811001

If the applicant is a corporate body, give the country/state of its incorporation — **Italy**

4. Title of the invention — **Antigens**

5. Name of your agent *(if you have one)* — **Carpmaels & Ransford**

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

43 Bloomsbury Square
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Patents ADP number *(if you know it)* — **83001**

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body

See note (d))

Yes

ANTIGENS

This invention relates to antigenic proteins from *Chlamydia trachomatis*. In particular, it relates to antigens which are recognised by antibodies from chronically infected or convalescent patient sera.

BACKGROUND

The *Chlamydia* are obligate intracellular parasites of eukaryotic cells which are responsible for endemic sexually transmitted infections, trachoma, infectious pneumonitis, and various other disease syndromes. They occupy an exclusive eubacterial phylogenic branch, having no close relationship to any other known organisms – they are classified in their own order (*Chlamydiales*) which contains a single family (*Chlamydiaceae*) which in turn contains a single genus (*Chlamydia*). Four chlamydial species are currently known – *C.trachomatis, C.pneumoniae, C.pecorum* and *C.psittaci* [*eg.* see reference 1]. A genome sequence of *C.trachomatis* (serovar D) has recently been published [2].

The human serovariants ("serovars") of *C.trachomatis* are divided into two biovariants ("biovars"). Serovars A-K elicit epithelial infections primarily in the ocular tissue (A-C) or urogenital tract (D-K). Serovars L1, L2 and L3 are the agents of invasive lymphogranuloma venereum (LGV).

Although chlamydial infection itself causes disease, it is thought that, in some patients, the severity of symptoms is due, in fact, to an aberrant host immune response. Failure to clear the infection results in persistent immune stimulation and, rather than helping the host, this results in chronic infection with severe consequences, including sterility and blindness [3].

In addition, the protection conferred by natural chlamydial infection, is usually incomplete, transient, and strain-specific.

Due to the serious nature of the disease, there is a desire to provide suitable vaccines. These may be useful (a) for immunisation against chlamydial infection or against chlamydia-induced disease (prophylactic vaccination) or (b) for the eradication of an established chronic chlamydial infection (therapeutic vaccination). Being an intracellular parasite, however, the bacterium can generally evade antibody-mediated immune responses.

Various antigenic proteins have been described for *C.trachomatis*, and the cell surface in particular has been the target of detailed research [*eg.* 1,4]. These include, for instance, pgp3 [5,6,7], MOMP [8], Hsp60 (GroEL) [9] and Hsp70 (DnaK-like) [10]. Not all of these have

According to a further aspect, the invention provides nucleic acid encoding the proteins and protein fragments of the invention. Nucleic acid having sequence identity to this nucleic acid is also provided. Depending on the particular nucleic acid, the degree of identity is preferably greater than 50% (*eg.* 65%, 80%, 90%, 95%, 98%, 99% or more).

Furthermore, the invention provides nucleic acid which can hybridise to this nucleic acid, preferably under "high stringency" conditions (*eg.* 65°C in a 0.1xSSC, 0.5% SDS solution).

Fragments of this nucleic acid are also provided. The fragments should comprise at least *n* consecutive nucleotides from the sequences and, depending on the particular sequence, *n* is 10 or more (*eg.* 12, 14, 15, 18, 20, 25, 30, 35, 40 or more).

It should also be appreciated that the invention provides nucleic acid comprising sequences complementary to those described above (*eg.* for antisense or probing purposes).

Nucleic acid according to the invention can, of course, be prepared in many ways (*eg.* by chemical synthesis, from genomic or cDNA libraries, from the organism itself *etc.*) and can take various forms (*eg.* single stranded, double stranded, vectors, probes *etc.*).

In addition, the term "nucleic acid" includes DNA and RNA, and also their analogues, such as those containing modified backbones, and also peptide nucleic acids (PNA) *etc.*

According to a further aspect, the invention provides vectors comprising nucleic acid of the invention (*eg.* expression vectors) and host cells transformed with such vectors.

According to a further aspect, the invention provides compositions comprising protein, antibody, and/or nucleic acid according to the invention. These compositions may be suitable as immunogenic compositions (including vaccines), for instance, or as diagnostic reagents.

The invention also provides nucleic acid, protein, or antibody according to the invention for use as medicaments (*eg.* as vaccines) or as diagnostic reagents. In particular, the invention provides protein 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, or 55 (as set out in Table II on page 15) for use as a chlamydial immunogen. Whilst it is believed that some of the proteins described in Table II may be known *per se*, they have not been disclosed as being immunogenic.

The invention also provides the use of nucleic acid, protein, or antibody according to the invention in the manufacture of (i) a medicament for treating or preventing infection due to *Chlamydia*; (ii) a diagnostic reagent for detecting the presence of *Chlamydia* or of antibodies

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the annotated reference 2D electrophoretic EB map, also indicating the positions of the immunoreactive protein spots, labelled 1-55. Groups of spots which appear to be an isolectric series of the same protein are encircled together and classified under the same identification number.

Figure 2 shows typical immunoblots. The whole map area is shown. Major known immunogens are marked for easier comparison. For other spot identification, refer to Figure 1 and table II. Blot A is from PID patient JO51 (MIF titre 256), and has a serum dilution 1:5000. Blot B is from patient JO35 (MIF titre 64) affected by secondary sterility, and has a serum dilution 1:2500. Blot C is similar to blot B, but is from patient JO52. Blot D is from PID patient JO31 (MIF titre 256), and gas a serum dilution 1:5000.

EXAMPLES

Human sera

Sera (Table I) were obtained from women who had responded to a chlamydial infection of the genital tract. The seventeen sera (A...Q) were obtained from 4 cases of lower genital tract infection and 13 laparoscopically-confirmed cases of PID (pelvic inflammatory disease), including 2 cases of secondary sterility. All sera were positive for a standard microimmunofluorescence test (MIF) with purified *C.trachomatis* L2 elementary bodies [11], and confirmed as *C.trachomatis* immune sera by an ELISA test with the plasmid-encoded pgp3 antigen [5].

A group of 10 seronegative control sera from healthy blood donors was tested by immunoblotting in the same way, and using the same dilutions as for patient sera, in order to exclude the occurrence of non-specific reactions.

Most sera were obtained from the Chlamydia collection of the Biobanque de Picardie (Amiens, France). Some PID and control sera from healthy blood donors were obtained from the Ospedale Policlinico S.Orsola (Bologna, Italy).

Preparation of protein samples

Purified chlamydial cells were obtained as described in reference 12, by growing *C.trachomatis* strain L2/343/Bu in Vero cell cultures according to standard procedures, followed by two cycles of density gradient centrifugation [13]. The average protein concentration of the purified elementary body (EB) preparation was determined using a biuret

Ponceau S in 3% (w/v) trichloroacetic acid for 3 minutes and the positions of selected anchor spots were marked on the blot to assist matching of the immunoblots with the silver stained map. Immunoreactive spots were detected by overnight incubation at room temperature with patient sera (1500-5000x dilutions), followed by incubation with rabbit anti-human IgGs conjugated with peroxidase (Cappel, 7000x dilution), and detection with a chemiluminescence based kit (Pharmacia Amersham Biotech).

Typically, six identical 2D maps were prepared in parallel for each experiment – five were blotted onto nitrocellulose and one was stained with silver nitrate for subsequent correlation with the immunoblots and computer-assisted matching to the reference map.

The spot signals on the immunoblot almost always corresponded to a spot on the silver stained gel. However, in at least two instances (spots 13 and 14 in Figure 1), immunoblot analysis detected protein spots which were not visible in the silver stained map. This shows that this technique has a superior sensitivity and should be taken into consideration as a valuable tool also for systematic proteomics studies.

To assist matching of the immunoblot with the reference map shown in Figure 1, the nitrocellulose blots were marked with a number of internal "anchor" spots using transient Ponceau Red staining. After incubation with the sera and detection of bound antibodies by chemiluminescence, the immunoblot images were matched to the reference map and spots were assigned the corresponding pI and MW coordinates (see Table II). When the position and shape of the spot (or isoelectric series of spots) coincided with a previously-identified EB antigen, an immune response against such antigen was recorded. In all other cases the immunoblot spot was identified by the MW and pI coordinates taken at the baricentre of the stained area (or the coordinate range, in the case of complex spot patterns). It will be appreciated that the MW and pI values are determined electrophoretically, and may have a potential average error of +/-10%. The higher MW measurements will tend to be less accurate.

While control blots were totally blank, patient blots showed individually different patterns comprising a number of spots, which varied from 2 to 28, with an average of around 15 (see Table II). The number of immunoreactive spots had did not correlate with the serum MIF titres (see Tables I and II), so blot patterns appear to reflect a real individual variation in humoral responses, and not just the difference of antibody titres. This was also confirmed by comparing the results of each serum at various dilutions.

Coomassie Brilliant Blue R250 in 50% aqueous methanol for 5 minutes, and de-stained in 40% methanol, 10% acetic acid. Membranes were dried at 37°C and stored at –20°C for further analysis. Selected protein spots were cut out and submitted to amino acid sequencing by Edman degradation using an automatic Protein/Peptide Sequencer (mod 470A; Applied Biosystem Inc.) connected on-line with a phenylthiohydantoin-amino acid analyser model 120A and a control/Data Module model 900A (Applied Biosystems Inc.). Typically 3 or 4 equivalent spots from similar blots were used, according to the estimated relative molar amount of protein in the spot.

The results of the sequencing are shown in Table III on page 16.

Computer analysis of sequences

Using the N-terminal sequence data, database searches for protein similarity were performed using the BLAST program [21] available from NCBI [*http://www.ncbi.nlm.nih.gov*] and programs of the GCG software (Wisconsin Package Version 9.0) [22]. Theoretical pI and MW values were calculated by the pI/MW computer program available from the ExPASy internet server [*http://www.expasy.ch*].

In addition to the usual databases, the genomic sequencing data of the *C.trachomatis* D/UW-3/Cx strain provided by the Chlamydia Genome Project [*http://chlamydia-www.berkeley.edu:4231*] was searched. Although the present study used a *C.trachomatis* serovar L2 strain (lymphogranuloma biovar), which has a different pathogenicity phenotype, several protein sequences could be safely correlated to the serovar D genes.

These searches with N-terminal data allowed the correlation of seven immunoreactive spots to known sequences (in addition to the seven noted above):

- Spot 15: predicted to be a periplasmic peptidase (currently annotated in the serovar D genomic database as *htrA*).
- Spots 18 & 46: predicted to be an outer membrane protein (currently annotated in the genomic database as *ompB*).
- Spot 21: Although the amino acid sequence does not match any previously-described proteins, it shows homology to an internal sequence from EF-Tu. This protein may be a breakdown or processing product of EF-Tu, or a variant.
- Spot 24: the RNA polymerase alpha subunit (*rpoA*)

The reason why this protein appears as two distinct electrophoretic species was not investigated, but a spot shift of this type is usually associated to a variation of amino acid composition, either due to amino acid sequence variation, and/or to true or artefactual derivatisation of some amino acid residues.

Five patients showed reactivity to this protein, demonstrating that it is immunogenic in humans as a consequence of chlamydial infection.

Spot 25

This spot is believed to be an aminopeptidase, based on its MW/pI position and on its N-terminus sequence (both in comparison with the published serovar D sequence *pepA*). Four patients showed reactivity to this protein, demonstrating that it is immunogenic in humans as a consequence of chlamydial infection.

Spot 38

This spot is believed to be a GTP-binding protein, based on its MW/pI position and on its N-terminus sequence (both in comparison with the published serovar D sequence *ychF*). Two patients showed reactivity to this protein, demonstrating that it is immunogenic in humans as a consequence of chlamydial infection.

Spot 15

This spot is believed to be a stress-induced protease, based on its MW/pI position and on its N-terminus sequence (both in comparison with the published serovar D sequence *htrA*). Seven patients showed reactivity to this protein, demonstrating that it is immunogenic in humans as a consequence of chlamydial infection.

Spot 8

Nine patients showed reactivity towards protein spot 8, which could not be characterised by N-terminal sequencing. It does, however, have the following 'constellation type 2' amino acid composition (molar percentages):

aa	%	aa	%	aa	%	aa	%
Ala	6.5	Gly	22.5	Lys	3.7	Ser	13.7
Arg	3.5	His	0.5	Met	0.5	Thr	5.1
Asx	8.4	Ile	3.7	Phe	2.8	Tyr	2.2
Glx	12.5	Leu	6.7	Pro	3.4	Val	4.3

family); and seven sera (41%) recognised spot 12 (the ribosomal protein L7/L12). In the group of sera used in this study, 12/17 (70.6%) reacted with at least one of these five antigens and, including the hsp60 and hsp70 antigens, all sera had antibodies reacting with between 1 and 7 (average 3.7) chlamydial proteins which have homologs in other bacteria.

Theories which postulate a role for immunological sensitisation mechanisms in chlamydial pathology, as described for the hsp60 GroEL-like antigen [29], should in fact be extended to several other common bacterial antigens, which may be immunogenic in other bacterial infections. For instance the protein elongation factor EF-Tu is immunogenic during the acute phase of infection with *Haemophilus influenzae*, and both L7/L12 and the HtrA stress-induced protease homologues are immunogenic in *Brucella* infections. In the case of EF-Tu, the abundance of this protein in the bacterial cell may favour its "visibility" by the immune system. It should be noted, however, that EF-Tu has been described as associated to outer membrane and periplasmic cell fractions [30], and more recently data suggest that EF-Tu, in addition to its function in peptide elongation, has also a chaperone activity implicated in protein folding and protection from stress [31]. Particularly intriguing is the response to the L7/L12 ribosomal protein, since in *Brucella melitensis* infections the homologous L7/L12 antigen induces a DTH cell-mediated response [27]. Furthermore vaccination of BALB/c mice with L7/L12 was shown to give protection against infection by *B.abortus* [32]. The unexpected finding that antibodies to L7/L12 are fairly frequent in patients infected by *C.trachomatis* suggests that perhaps further attention should be paid to this antigens also in chlamydia-induced disease.

Spots 5, 6, 7, & 9

These proteins, whilst not yet correlated with any available genome sequence, and not yet having been sequenced, are of obvious interest given their prevalence (>50%) in the sera tested.

It will be understood that the invention is described above by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

TABLE II – PATIENT REACTIVITY WITH PROTEIN SPOTS

Spot #	pI	MW	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	FREQ
1	complex		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	17
2	5.2-5.3	59.7	+	+	+	+	+	+	+	+	+	+	+			+	+	+	+	15
3	4.6-4.9	40	+	+	+	+	+	+	+		+	+		+		+	+		+	13
4	4.92-5.04	70.5	+	+	+	+	+	+			+	+	+		+				+	11
5	5.09	36.6	+	+	+	+	+	+			+	+				+		+	+	11
6	6.34	46.2-50	+			+	+	+			+	+	+			+		+	+	10
7	6.59	46.2-50.2	+			+	+	+			+	+	+			+		+	+	10
8	4,96	36,6	+		+		+	+			+	+				+		+	+	9
9	6.36	37.7-39.4	+	+			+	+			+	+	+					+	+	9
10	5.44-5.64	42.2	+	+	+		+		+				+					+	+	8
11	6.66	26.1		+			+	+			+		+			+	+		+	8
12	4.80	15.8		+		+	+				+		+	+						6
13	6.1	37.4-39.2	+	+			+						+			+		+	+	7
14	6.24	47.9	+				+	+			+	+	+					+		7
15	5.89	48.4	+	+	+		+	+					+					+		7
16	6.15	46-50	+				+				+	+	+					+	+	7
17	5.92	25.3		+			+	+								+	+		+	6
18	5.08	34.09			+	+		+					+						+	5
19	5.14-5.28	69	+		+	+		+					+							5
20	5.44	26.2		+			+									+	+		+	5
21	5.27	40.5		+	+	+							+						+	5
22	4.81	46.3	+		+								+	+		+				5
23	4.97	34.2			+			+					+							4
24	5.32	40.5			+	+							+						+	4
25	5.97	47.6	+	+														+	+	4
26	5.68	48.6	+					+					+						+	4
27	6.29-6.42	124.5	+				+					+						+		4
28	5.39	25.5					+	+								+				3
29	5.1	28.7					+				+					+				3
30	4.8	36.7			+		+												+	3
31	5.43	40.4				+							+						+	3
32	5.2-5.37	62.4			+			+					+							3
33	6.64	25.4					+				+									2
34	4.79	28.1				+													+	2
35	4.82	29.5				+													+	2
36	6.55	37.5					+					+								2
37	5.14	40.3		+															+	2
38	5.23	40.1																+	+	2
39	4.69	45.7		+										+						2
40	6.89	50										+						+		2
41	6.39-6.55	105	+				+													2
42	4.57	20.3									+									1
43	4.72	26.5					+													1
44	7.6	26.95											+							1
45	6.9	29.7														+				1
46	5.19	33.4				+														1
47	6.99	35.8											+							1
48	6.54	35.8											+							1
49	5.44	39.0																	+	1
50	5.37	41.0																	+	1
51	7.59	42.6											+							1
52	8.73	49.2					+									+				1
53	7.98	49.4			+															1
54	7.4	50.2											+							1
55	7.4	51.5											+							1

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15. The use of a protein according to any one of claims 1 to 4, an antibody according to claim 5, and/or nucleic acid according to any one of claims 6 to 9, in the manufacture of a medicament for treating or preventing infection due to *Chlamydia*.

16. The use of a protein according to any one of claims 1 to 4, an antibody according to claim 5, and/or nucleic acid according to any one of claims 6 to 9, in the manufacture of a diagnostic reagent for detecting the presence of *Chlamydia* or of antibodies raised against *Chlamydia*.

17. The use of a protein according to any one of claims 1 to 4, an antibody according to claim 5, and/or nucleic acid according to any one of claims 6 to 9, in the manufacture of a reagent which can raise antibodies against *Chlamydia*.

18. A method of treating a patient, comprising administering to the patient a therapeutically effective amount of a protein according to any one of claims 1 to 4, an antibody according to claim 5, and/or nucleic acid according to any one of claims 6 to 9.

19. A process for producing a protein according to any one of claims 1 to 4, comprising the step of culturing a host cell according to claim 11 under conditions which induce protein expression.

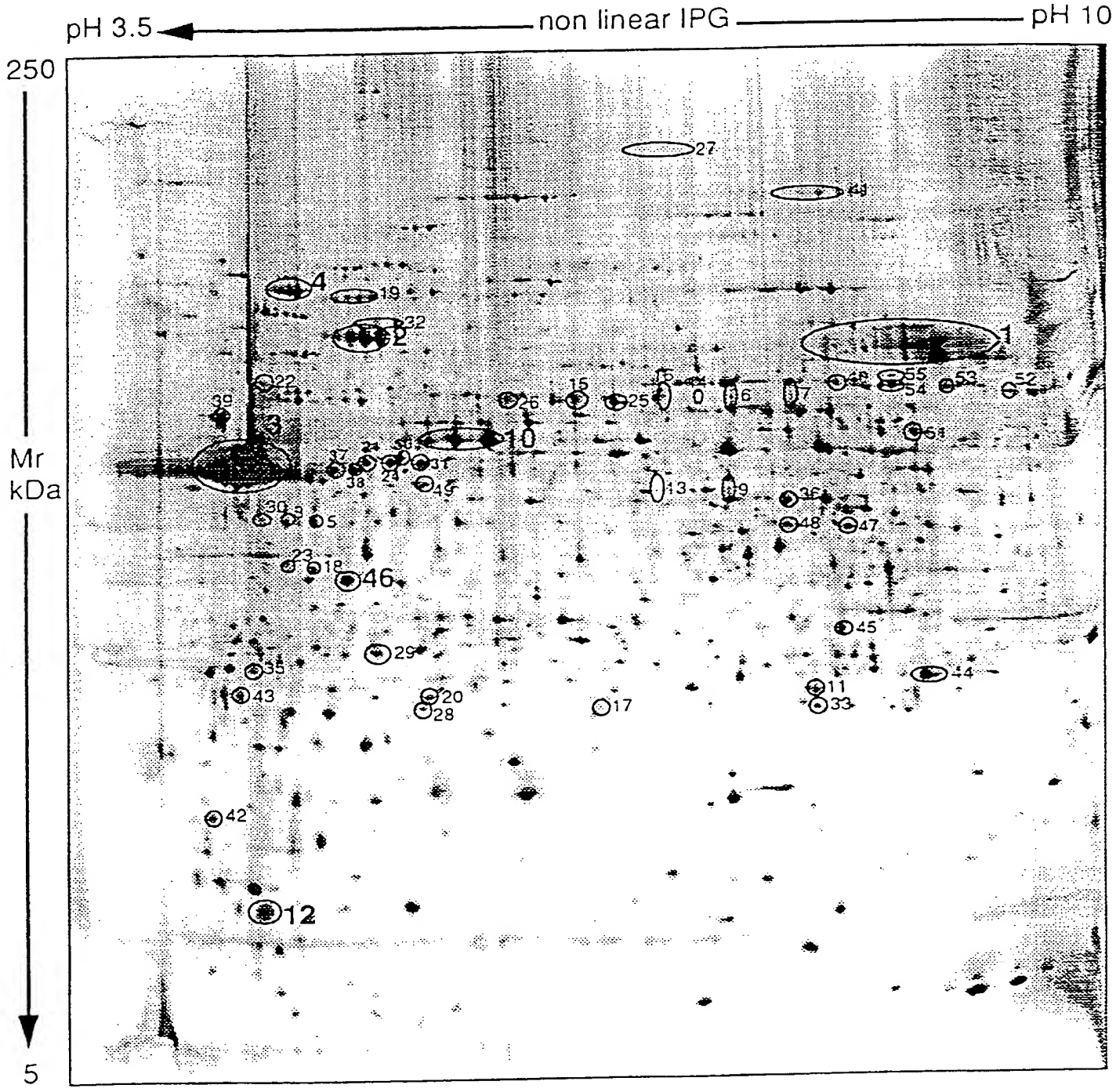
20. A process for producing a protein according to any one of claims 1 to 4 or nucleic acid according to any one of claims 6 to 9, wherein the protein or nucleic acid is synthesised in part or in whole using chemical means.

21. A process for detecting nucleic acid according to claim any one of claims 6 to 9, comprising the steps of: (a) contacting a nucleic acid probe with a biological sample under hybridising conditions to form duplexes; and (b) detecting said duplexes.

22. A process for detecting a protein according to any one of claims 1 to 4, comprising the steps of: (a) contacting an antibody according to claim 5 with a biological sample under conditions suitable for the formation of an antibody-antigen complexes; and (b) detecting said complexes.

23. A process for detecting an antibody according to claim 5, comprising the steps of: (a) contacting a protein according to any one of claims 1 to 4 with a biological sample under conditions suitable for the formation of an antibody-antigen complexes; and (b) detecting said complexes.

24. A kit comprising reagents suitable for use in a process according to any one of claims 21 to 23.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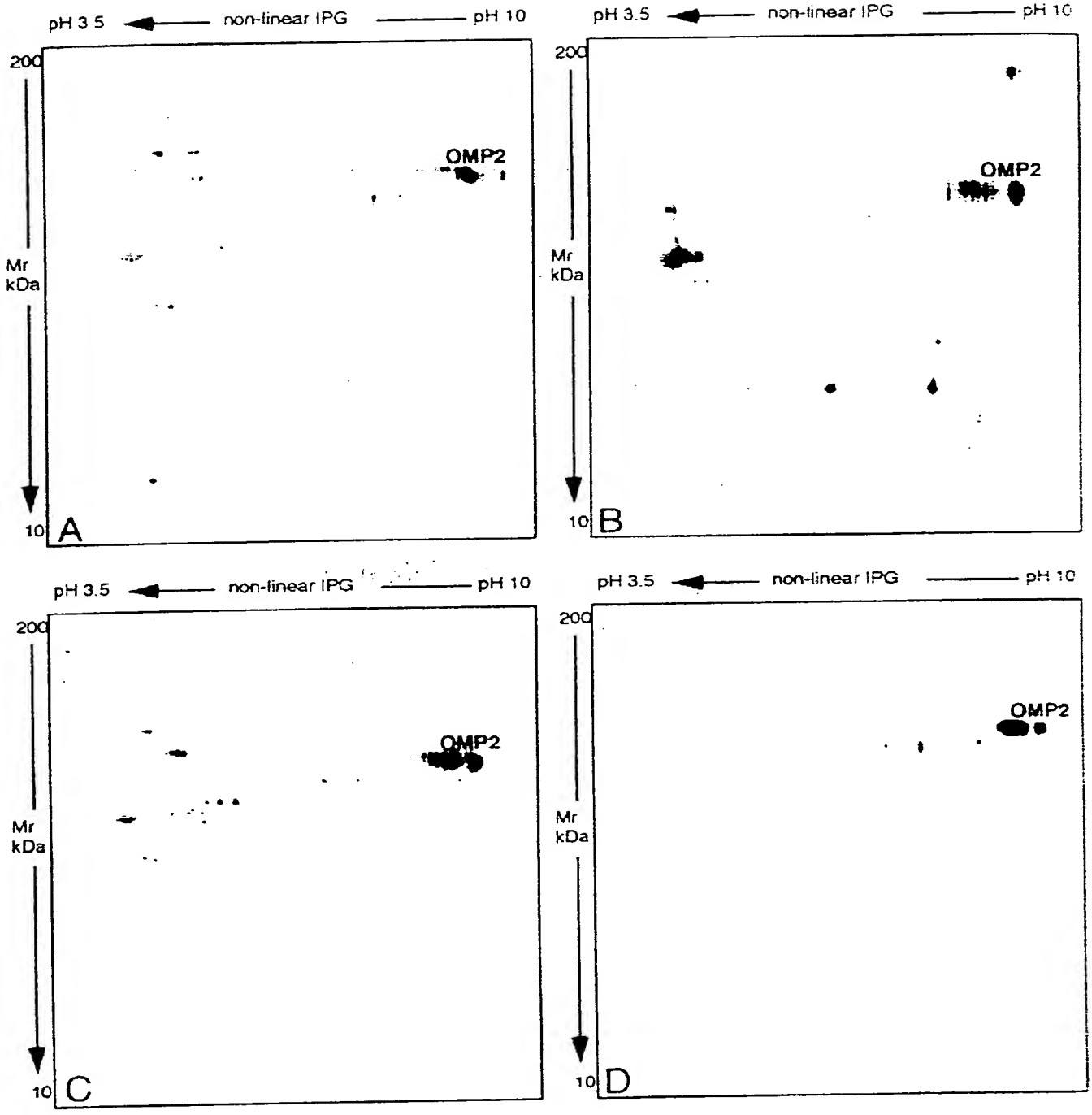

HP Deskjet 3700

Fig 2

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